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Review

The biology of cancer testis antigens: Putative function, regulation and therapeutic potential

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ABSTRACT

Cancer testis antigens (CTA) are a large family of tumor-associated antigens expressed in human tumors of different histological origin, but not in normal tissues except for testis and placenta. This tumor-restricted pattern of expression, together with their strong *in vivo* immunogenicity, identified CTA as ideal targets for tumor-specific immunotherapeutic approaches, and prompted the development of several clinical trials of CTA-based vaccine therapy. Driven by this practical clinical interest, a more detailed characterization of CTA biology has been recently undertaken. So far, at least 70 families of CTA, globally accounting for about 140 members, have been identified. Most of these CTA are expressed during spermatogenesis, but their function is still largely unknown. Epigenetic events, particularly DNA methylation, appear to be the primary mechanism regulating CTA expression in both normal and transformed cells, as well as in cancer stem cells. In view of the growing interest in CTA biology, the aim of this review is to provide the most recent information on their expression, regulation and function, together with a brief summary of the major clinical trials involving CTA as therapeutic agents. The pharmacologic modulation of CTA expression profiles on neoplastic cells by DNA hypomethylating drugs will also be discussed as a feasible approach to design new combination therapies potentially able to improve the clinical efficacy of currently adopted CTA-based immunotherapeutic regimens in cancer patients.

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1. Introduction

The search for tumor-associated antigens (TAA) capable to induce a tumor-directed immune response and the development of cancer vaccines targeting these TAA have been a major effort for the tumor immunology community in the past decades. Most recently, significant progresses have been made in the identification of human TAA recognized by T cells. In the early

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1990s, Boon et al. reported the first successful cloning of a human tumor antigen, termed melanoma antigen-1 (MAGE-1), which elicited an autologous cytotoxic T-lymphocyte (CTL) response in a melanoma patient (van der et al., 1991). Further studies demonstrated that MAGE-1 (later renamed MAGE-A1) was expressed in various cancers of different histological origin but not in normal tissues except testis and placenta (De Plaen et al., 1994; Zendman et al., 2003). The approach that led to MAGE-A1 discovery consisted in expanding CTL from the peripheral blood of cancer patients through their *in vitro* stimulation with autologous tumor cells. Resulting CTL clones were then restimulated with cells transfected with cDNA libraries constructed from autologous tumor cells in order to identify the gene encoding the relevant antigen (van der et al., 1991). This strategy, termed T-cell epitope cloning, successfully identified other tumor antigens including MAGE-A2, MAGE-A3, BAGE and GAGE-1 (Boel et al., 1995; De Backer et al., 1999; Chomez et al., 2001; Gaugler et al., 1994). Shortly after the discovery of TAA through the T-cell epitope cloning method, Pfreundschuh et al. developed a serological approach based on the immunoscreening of tumor cDNA expression libraries with antibodies from patients, rather than with T cells (Sahin et al., 1997). This technology, termed serological analysis of cDNA expression libraries (SEREX) has enabled the discovery of several novel immunogenic TAA with restricted expression in testis and various cancer types, including synovial sarcoma/X breakpoint 2 (SSX-2) (Tureci et al., 1998a) and the highly immunogenic tumor antigen New York oesophageal squamous cell carcinoma 1 (NY-ESO-1) (Chen et al., 1997). Next to these immunological methods, a multitude of tumor genes with a cancer/testis restricted expression profile has been identified using differential gene expression techniques comparing mRNA pools of tumor versus normal, or testis versus other tissues. Several techniques were used based on this principle, including differential display, cDNA oligonucleotide array analysis and bioinformatic analysis. To encompass the growing list of genes expressed in malignancies of various histotypes, but not in normal tissue except testis and placenta, a nomenclature for this category of tumor antigens was needed; Thus, the term cancer testis antigen (CTA) was coined by Old and Chen (Chen et al., 1997; Old and Chen, 1998). So far, at least 70 families of CTA with over 140 members have been identified and recently listed in a database established by the Ludwing Institute for Cancer Research (http://www.cta.Incc.br/) (Almeida et al., 2009).

CTA can be divided in those that are encoded on the X chromosome, the X-CTA genes, and those that are not, the non-X-CTA genes (Simpson et al., 2005). It has been estimated that 10% of genes on the X chromosome belong to X-CTA families. The X-CTA genes represent more than half of all CTA and often constitute multigene families organized in well-defined clusters along the X chromosome, where the different members are arranged into complex direct and inverted repeats. A few examples are the Xp11 region, which contains all genes of the SSX family and GAGE/PAGE/XAGE super-families, and the Xq24-q28 region, which bears the highest density of CTA genes, including MAGE-A, MAGE-C and NY-ESO-1 multigene families (Simpson et al., 2005; Zendman et al., 2003). The genes for the non-X CTA, on the other hand, are distributed throughout the genome and are mostly single-copy genes (Simpson et al., 2005) (Table 1).

1.1. *CTA expression in normal and cancer tissues*

In normal testis, X-CTA genes are expressed primarily on the spermatogonia that are proliferating germ cells, while non-X CTA are expressed in later stages of germ-cell differentiation, such as on spermatocytes (Simpson et al., 2005). In addition to testicular expression, MAGE-A3, MAGE-8, MAGE-A10, XAGE-2 and XAGE-3 have been found to be expressed in placenta (Caballero and Chen, 2009). Some somatic tissues such as pancreas, liver and spleen have been demonstrated to express mRNA of several CTA. However, based on quantitative RT-PCR data, mRNA levels of CTA genes in somatic tissues are usually <1% of their expression in testis (Caballero and Chen, 2009; Scanlan et al., 2004).

CTA are widely and variably distributed among tumors of different histotypes. The available data are mainly based on the analysis of their transcripts, and demonstrate that the expression of CTA varies greatly among tumor types. According to RT-PCR analysis, members of different families and super-families of CTA are largely expressed in melanoma, bladder and non-small cell lung cancers, moderately expressed in breast and prostate cancers, poorly expressed in kidney and colon cancers (Scanlan et al., 2002, 2004) and in hematologic malignancies, such as primary effusion lymphoma and Hodgkin's and non-Hodgkin's lymphomas (Calabro et al., 2005; Gattei et al., 2005). The CTA MAGE-A1, MAGE-A3, NY-ESO-1, SSX-2 and SSX-4 appear to be the most frequently expressed, while BAGE, GAGE-A1 and SCP-1 are rarely expressed (Scanlan et al., 2002). In particular, the frequency of NY-ESO-1 mRNA expression was 17–42% in melanomas (Chen et al., 1997; van der et al., 2002; Vaughan et al., 2004; Sigalotti et al., 2002b), 32%–80% in bladder cancers (Kurashige et al., 2001; Scanlan et al., 2004), 27% in non-small-cell lung carcinomas (NSCLC) (Gure et al., 2005), and 2–10% in colon cancers (Li et al., 2005; Mashino et al., 2001), while CTA have not been detected in renal cell carcinomas and lymphomas (Chen et al., 1997). Similarly, MAGE-A3 has been detected in 57%–76% of melanomas (Sigalotti et al., 2002b; Brasseur et al., 1995; Gaugler et al., 1994; Roeder et al., 2005), 35%–60% of NSCLC (Scanlan et al., 2000; Melloni et al., 2004), 57% of bladder cancers (van der et al., 2002), in 75% of squamous esophageal cancers (Weinert et al., 2009) and in 42% of hepatocarcinomas (Chen et al., 1999) (Table 2). In contrast BAGE mRNA has been found expressed in 21% of hepatocarcinomas (Kobayashi et al., 2000), in 17%–20% of NSCLC (Melloni et al., 2004; Tajima et al., 2003), in 14%–28% of melanomas (Boel et al., 1995; Ruault et al., 2002; Sigalotti et al., 2002b) and in 15% of bladder cancers (Scanlan et al., 2002). Interestingly, some reports suggest that multiple CTA tend to be co-expressed in the same neoplastic lesion (Gure et al., 2005). In this respect, a study conducted to assess CTA expression in a panel of breast cancers and melanomas revealed that whereas no expression of investigated CTA was detectable in 47% of breast tumors and 26% of melanomas, in 40% of breast carcinomas and 65% of melanomas a concomitant expression of at least 3 CTA was observed (Sahin et al., 1998). Similarly, while 11 of 33 lung cancers showed no expression of any of the CTA typed, 19 of 22 CTA-positive lung tumors expressed at least 2 CTA and 13 of 22 expressed at least 3 CTA (Scanlan et al.,

2000). Despite these suggestive observations, a study evaluating the expression of MAGE-A2, -A3 and -A4, GAGE-1–6, BAGE, NY-ESO-1 and PRAME in unrelated metastatic lesions from 53 melanoma patients failed to identify any statistically significant association between the expression of investigated CTA (Sigalotti et al., 2002b).

In addition to mRNA analysis by RT-PCR, the expression of few CTA has been investigated at protein level by immunohistochemistry (IHC) analysis, showing a good correlation between protein and mRNA data (Jungbluth et al., 2000, 2001; Landry et al., 2000; Rimoldi et al., 2000). The use of IHC allowed to investigate the intratumoral distribution of CTA, thus revealing what can be defined a hallmark of CTA expression in cancer: its intratumoral heterogeneity. Indeed, usually less than 50% of neoplastic cells are stained by anti-MAGE-A1, -NY-ESO-1 or -SSX monoclonal antibodies in the majority of CTA-positive lesions from tumors of different histotypes (dos Santos et al., 2000; Jungbluth et al., 2000, 2001; Maio et al., 2003a).

2. Regulation of CTA expression

Thus far, epigenetic events appear to represent the unique mechanism regulating CTA expression both in normal and neoplastic cells (Karpf and Jones, 2002). The term "epigenetic" refers to heritable changes in gene expression that do not derive from alterations of the nucleotide sequence of genomic DNA (Antequera and Bird, 1999; Klose and Bird, 2006; Sigalotti et al., 2007), and DNA methylation and histone post-translational modifications represent the most widely characterized epigenetic factors controlling CTA expression (Figure 1).

2.1. *DNA methylation*

DNA methylation is a commonly occurring modification of DNA that leads to silencing of gene expression and refers to the covalent addition of a methyl group, catalyzed by DNA methyltransferases (DNMTs), to the 5-carbon position of cytosine bases in the cytosine-guanine dinucleotides (CpG) of mammalian DNA strands. DNA methylation can cause gene silencing either by directly interfering with the binding of specific transcription factors to the DNA (Watt, 1998) or by binding methyl-CpG-binding proteins (MBDs), which prevent gene expression by recruiting chromatin remodeling co-repressor complexes (Jones et al., 1998; Wade et al., 1999; Wade, 2001; Zhang et al., 1999). So far, all CTA genes studied have methylated promoters in normal,

Table 1 – List of selected known X- and non-X CTA.[a]

	CTA	Number of genes	Chromosome location	Method of identification	References
	MAGE family				
X-CTA	MAGE-A	12	Xq28	T-cell epitope cloning/ molecular methods[b]	(van der et al., 2002; Ohman and Nordqvist, 2001)
	MAGE-B	6	Xp21.3	Molecular methods/RDA[c]	(Lucas et al., 2000; Lurquin et al., 1997; Muscatelli et al., 1995)
	MAGE-C	3	Xp26-27	SEREX[d]/RDA	(Lucas et al., 1998, 2000)
	GAGE/PAGE/XAGE superfamily				
	GAGE-A	8	Xp11.23	T-cell epitope cloning/ molecular methods	(De Backer et al., 1999; Gjerstorff and Ditzel, 2008; van den et al., 1995)
	GAGE-B	8	Xp11.23	RDA/database mining	(Chen et al., 1998; Gjerstorff and Ditzel, 2008)
	PAGE	5	Xp11.23	RDA/database mining	(Brinkmann et al., 1998, 1999; Chen et al., 1998)
	XAGE	5	Xp11.21–11.22	Database mining	(Brinkmann et al., 1999; Liu et al., 2000; Wang et al., 2001; Zendman et al., 2002)
	SSX family				
	SSX	5	Xp11.2	SEREX	(Tureci et al., 1998a; Gure et al., 1997)
	NY-ESO family				
	CTAG	3	Xq28	RDA/SEREX	(Chen et al., 1997; Lethe et al., 1998)
	Non-familial				
	CAGE	1	Xp22.11	SEREX	(Cho et al., 2002)
	HOM-TES-85	1	Xq23	SEREX	(Tureci et al., 2002)
	SAGE	1	Xq26	RDA	(Martelange et al., 2000)
Non-X CTA	BAGE	5	21p11.1	CTL epitope cloning	(Boel et al., 1995)
	BORIS	1	20q13.2	Molecular methods	(Loukinov et al., 2002)
	CT9/3BRDT	1	1p22.1	Database mining	(Scanlan et al., 2000)
	HAGE	1	6q12-13	RDA	(Martelange et al., 2000)
	OY-TES-1	1	12p12-13	SEREX	(Ono et al., 2001)
	SCP-1	1	1p12-p13	SEREX	(Tureci et al., 1998b)
	SPO11	1	20q13.2-q13.3	Database mining	(Koslowski et al., 2002)

a CTA selection is based on their wide characterization.
b Molecular methods include screening recombinant libraries with probes, exon trapping, electrophoretic mobility shift assays.
c Representative differential analysis.
d Serological analysis of cDNA expression libraries.

Table 2 – RT-PCR analysis of the expression of selected CTA in human tumors of different histotype.[a]

Tumor histotype	CTA				References
	MAGE-A1	MAGE-A3	NY-ESO-1	SSX-2	
Bladder	43[b]	57	32–80	44	(Scanlan et al., 2004; Kurashige et al., 2001; van der et al., 2002; Sharma et al., 2006)
Brain	16–40	56	20	6–29	(Liu et al., 2004; Oba-Shinjo et al., 2008; Lee et al., 2008; Sahin et al., 2000; Tureci et al., 1998a)
Breast	6–18	10	10	4	(Mashino et al., 2001; Scanlan et al., 2004; Otte et al., 2001)
Colon	30	20	2–10	12	(Mashino et al., 2001; Mori et al., 1996; Li et al., 2005)
Esophageal	53	75	24–33	0	(Mashino et al., 2001; Scanlan et al., 2004; Weinert et al., 2009; Fujita et al., 2004)
Head and neck	30–40	44–54	7–28	5–35	(Filho et al., 2009; Ries et al., 2009; Kienstra et al., 2003; Cuffel et al., 2010)
Liver	46–80	42	27–44	47	(Chen et al., 1999, 2000, 2001; Liu et al., 1999; Scanlan et al., 2004; Zhao et al., 2004; Nakamura et al., 2006; Sato et al., 2005)
Lung	36–49	35–60	27	15	(Gure et al., 2005; Scanlan et al., 2000; Melloni et al., 2004)
Melanoma	40–48	57–76	17–42	35	(Scanlan et al., 2004; Sigalotti et al., 2002b; Vaughan et al., 2004; van der et al., 2002; Gaugler et al., 1994; Brasseur et al., 1995; Tureci et al., 1998a)
Myeloma	20–52	52	7–36	12–23	(Andrade et al., 2008; Jungbluth et al., 2005; Pellat-Deceunynck et al., 2000; van et al., 1999; Atanackovic et al., 2009; Atanackovic et al., 2007; Taylor et al., 2005)
Neuroblastoma	66–36	33–49	36	ND[c]	(Grau et al., 2009; Soling et al., 1999)
Ovarian	28–55	37	30	10	(Gillespie et al., 1998; Scanlan et al., 2004; Zhang et al., 2010; Odunsi et al., 2003; Valmori et al., 2006)

a CTA selection is based on their wide characterization and frequency of expression.
b Percent of CTA-positive specimens.
c ND: not determined.

non-expressing somatic tissues, and are activated by demethylation during spermatogenesis (De Smet et al., 1999). The first evidence that CTA expression was regulated by DNA methylation was provided by Weber et al., who demonstrated that treatment with the DNA hypomethylating agent (DHA) 5-aza-2′-deoxycytidine (5-AZA-CdR) activated the *de novo* expression of MAGE-A1 gene in a human melanoma cell line (Weber et al., 1994). An in-depth analysis demonstrated that MAGE-A1 gene expression correlated with the methylation status of its promoter in neoplastic cells of different histotypes (De Smet et al., 1996). This was also true for different MAGE-A and NY-ESO members which expression was found to be invariantly associated with a hypomethylated status of their promoters in investigated tumors and cells lines (De Smet et al., 1996, 2004; Honda et al., 2004; Sigalotti et al., 2002b). The definitive proof that methylation status of CTA promoter is the leading molecular mechanism regulating CTA expression came from transfections experiments with reporter genes driven by CTA promoters, unmethylated or methylated *in vitro*, showing that promoter methylation was the only factor limiting CTA promoter activity in cancer cells (De Smet et al., 1995; Sigalotti et al., 2002b). Along this line, our recent studies demonstrated that promoter methylation status is directly responsible also for the highly heterogeneous intratumor expression of CTA that is frequently observed in human melanoma (Sigalotti et al., 2004). Interestingly, this promoter methylation heterogeneity was found to be further inherited at single-cell level, propagating the heterogeneous CTA expression profile to daughter generations (Fratta et al., 2010). The reported association between DNA hypomethylation of CTA promoters and CTA expression has been most recently confirmed on populations of putative melanoma stem cells, suggesting that epigenetic regulation might also be an important mechanism of CTA gene regulation in cancer stem cells (Sigalotti et al., 2008).

2.2. *Histone modifications*

Histone modifications also appear to play a role in the epigenetic regulation of CTA expression (Karpf, 2006; Sigalotti et al., 2007; Wischnewski et al., 2006). Histones are basic proteins containing flexible N-terminal tails protruding from the nucleosomes, which are extensively targeted by post-translational modifications, including acetylation and methylation. The acetylation status of histones is controlled by the balanced action of histone acetyltransferases (HAT), adding acetyl groups to the N-terminal lysine residues, and histone deacetylases (HDAC) playing the opposite role. The transcriptional regulation by acetylation is quite straightforward: acetylation of histones results in chromatin decompaction and gene transcription, while deacetylated chromatin associates with repressed genes (Iizuka and Smith, 2003). *In vitro* experiments have demonstrated that inhibition of HDAC by specific inhibitors (HDACi) associates to negligible effects on CTA expression in human malignancies, though a recent report showed some minor effect on MAGE-A genes expression and an up-regulated transcriptional activity of both methylated and unmethylated MAGE-A2 and -A12 promoters following HDACi treatment (Wischnewski et al.,

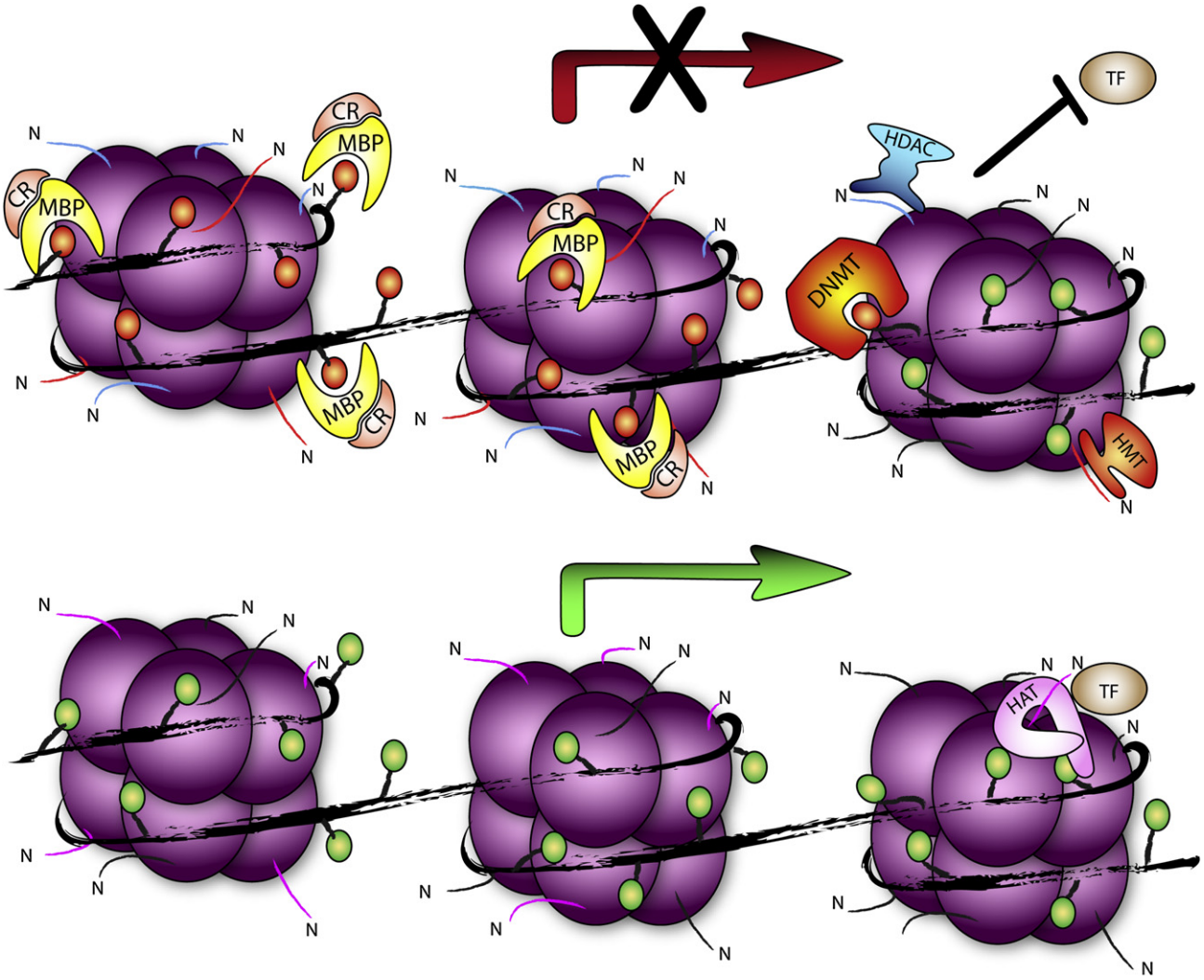

Figure 1 – Epigenetic regulation of CTA expression. Transcriptionally inactive CTA genes (upper panel) are characterized by the presence of methylated cytosines within the promoter region (orange circles), which is carried out and sustained by DNA methyltransferases (DNMT). Inhibition of CTA genes transcription may directly derive from methylated recognition sequence preventing the binding of transcription factors (TF), or may be a consequence of the binding of methyl-CpG-binding proteins (MBP), which prevent CTA gene expression by recruiting chromatin remodeling co-repressor complexes (CR). The presence within these CR of histone deacetylases (HDAC) that mediate deacetylation of histones (blue N-terminal tails), and of histone methyltransferases (HMT) that catalyze the methylation of histones (red N-terminal tails), causes the condensation of chromatin and makes it inaccessible to TF, thus playing an important accessory role to DNA methylation in repressing CTA expression (red arrow). On the other hand, demethylated CTA promoters (green circles) prevent the binding of MBP and CR, and are occupied by complexes including TF and histone acetyltransferases (HAT) that mediate acetylation of histones (pink N-terminal tails), finally resulting in a transcriptionally active CTA genes (green arrow).

2006). Confirming the major role of DHA in the functional modulation of CTA expression, the combined treatment of cancer cells of different histotypes with DHA and HDACi generally produced a modest synergistic effect that resulted in a 2–3 folds increase of CTA mRNA levels over DHA treatment alone (Sigalotti et al., 2007; Weiser et al., 2001a,b; Schrump and Nguyen, 2005).

Histone methylation also regulates gene expression. Histone methylation involves the addition of methyl groups to the N-terminal arginine and lysine residues, and unlike histone acetylation, is associated with both transcriptional activation and repression, depending on which residue is modified (Santos-Rosa and Caldas, 2005). Initial evidence for a potential role of histone methylation in CTA gene regulation was provided by Shinkai et al., who showed that the knockout of the histone methyltransferases (HMT) G9a and/or GLP, which target euchromatic loci and catalyze H3K9 dimethylation (H3K9me2), was sufficient *per se* to induce the expression of Mage-A genes in mouse embryonic stem cells (Tachibana et al., 2005, 2002). However, a recent study has demonstrated that the genetic knockdown of G9a and/or GLP in human colon cancer cells did not induce CTA expression, even though it reduced H3K9 methylation both globally and at CTA gene promoters (Link et al., 2009). Still, G9a inhibition synergized

with DHA to induce CTA expression in human colon cancer cells, thus suggesting that histone methylation likely plays an important accessory role to DNA methylation in repressing CTA expression (Link et al., 2009). Consistent with this data, 3-deazaneplanocin (DZNep), a potent inhibitor of the HMT EZH2, used in combination with both DHA and HDACi has proven effective in inducing GAGE gene expression in breast cancer cells as compared to each agent alone or in double combinations (DZNep plus DHA or DHA plus HDACi) (Sun et al., 2009).

3. Putative function of CTA

The biological role of CTA in both germline tissues and tumors remains poorly understood, but increasing evidences indicate that their expression could have a role in tumorigenesis.

Yeast two-hybrid studies have identified binding between MAGE-A1 protein and the transcriptional regulator SKI-interacting protein (SKIP), which participates in a wide variety of signaling pathways, including NOTCH1 pathway (Laduron et al., 2004). NOTCH1 is a transmembrane receptor that controls multiple cell differentiation processes during embryonic and adult life. Ligand binding to NOTCH1 induces the release of the intracellular domain of the receptor, NOTCH1-IC, which enters the nucleus and regulates the expression of several genes. One of the DNA-binding proteins that interact with NOTCH1-IC is the CBF1 that, in the absence of NOTCH1-IC, inhibits transcription by binding SKIP and a co-repressor complex containing HDAC1. Repression is relieved by the presence of NOTCH1-IC that binds to SKIP and CBF1, displaces the co-repressor complex, and activates transcription of target genes by recruiting HAT and other co-activators (Zhou et al., 2000). MAGE-A1 protein has been shown to counteract NOTCH1-IC transactivation by binding to SKIP and recruiting HDAC1, thus acting as a transcriptional repressor (Laduron et al., 2004). Although the function of MAGE-A1 in germline has not been elucidated so far, these evidences suggest that MAGE-A1 might repress the expression of genes required for differentiation during the spermatogenesis. Similarly, by recruiting HDAC1 to genes that remained to be defined, MAGE-A1 might be involved in the inhibition of cellular differentiation in cancer cells, thus contributing to tumorigenesis (Laduron et al., 2004; Simpson et al., 2005). Another yeast two-hybrid screen has identified MAGE-A4 as binding partner of the oncoprotein gankyrin, which has been implicated in the carcinogenesis of hepatocellular carcinoma (Higashitsuji et al., 2000; Nagao et al., 2003). MAGE-A4 was found to suppress the oncogenic activity of gankyrin through the action of a 107 amino acids peptide that is naturally cleaved from the C terminus of MAGE-A4. This binding was specific for MAGE-A4, because similar amino acid sequences from other MAGE-A family members did not bind to gankyrin. *In vitro* studies in nude mice have demonstrated that the binding of the C-terminal 107 amino acids of the MAGE-A4 to gankyrin inhibited the adhesion-independent growth of gankyrin-overexpressing cells. However, the mechanism underlying the effects of MAGE-A4 on gankyrin and anchorage-independent growth has not been fully elucidated yet (Nagao et al., 2003). MAGE-A11 was found to be involved in the regulation of androgen-receptor (AR) function by modulating its internal domain interactions. In the absence of androgen, MAGE-A11 interacts with the FXXLF motif in the N-terminal domain and stabilizes AR, leading to its accumulation in the cytoplasm. In the presence of ligand, the binding of MAGE-A11 to the AR N-terminal FXXLF motif increases AR transcriptional activity through the recruitment of the steroid receptor co-activator (SRC)/p160 family of co-activators (Bai et al., 2005). The MAGE-A11-induced stabilization of AR in the absence of hormone could account for the increased levels of AR frequently observed in clinical specimens of castration-recurrent prostate cancer (Chen et al., 2004; Koivisto et al., 1997; Visakorpi et al., 1995). Consistently, higher levels of expression of MAGE-11 mRNA were found to facilitate prostate cancer progression by enhancing AR-dependent tumor growth (Karpf et al., 2009). However, the functional role of MAGE-A11 in prostate cancer remains to be fully established.

Most recent studies further support the notion that the expression of MAGE genes in cancer cells might contribute to the malignant phenotype, suggesting, in addition, that it might have a role also in impairing the response to chemotherapeutic drugs. Along this line, *in vitro* experiments revealed that the transfection of ovarian cancer cell lines with MAGE-A2 and MAGE-A6 genes promoted cell growth and induced moderate paclitaxel and doxorubicin resistance, although the molecular mechanism of these effects has yet to be defined (Duan et al., 2003). MAGE-A2 was also shown to recruit HDAC3 to histones surrounding p53-binding sites, thereby down-regulating p53 transactivation functions and increasing resistance to etoposide treatment in short-term melanoma cell lines harboring wild-type-p53 (Monte et al., 2006).

Outside the MAGE gene families, antiapoptotic properties of GAGE-7 have been reported, as GAGE-7C was shown to render a human tumor-derived cell line resistant to apoptosis induced by interferon-γ (INF-γ) or Fas and also prevented killing induced by taxol and ionizing radiation (Cilensek et al., 2002). Consistent with this data, a recent study found the nuclear factor nucleophosmin (NPM)/B23 as a binding partner of GAGE-7C. In recent years, NPM/B23 has been recognized to play a major role in tumor development by virtue of its binding to many genes involved in cell proliferation, cancer, and cell cycle. Particularly, NPM/B23 inhibits DNA-binding and transcriptional activity of interferon regulatory factor-1 (IRF-1) tumor suppressor protein, which represent a key player in INF-γ -signaling pathway (Kular et al., 2009). *In vitro* analysis demonstrated that GAGE-7C was likely to bind to NPM/B23 without requiring IRF-1 since the interaction could be detected in cells in which the IRF-1 level was negligible. Upon induction of IRF-1 by IFN-γ treatment, IRF-1 was also found in this complex possibly due to binding of IRF-1 to NPM/B23. Consistently, overexpression of GAGE-7C resulted in stabilization and increased amounts of NPM/B23 in conjunction with decreased accumulation of IRF-1. This was accompanied by decreased levels of IRF-1 target genes, caspase-1 and caspase-7, thus suggesting that GAGE-7C overexpression might impair the apoptotic machinery and promoting cell survival (Kular et al., 2009).

With regard to SSX family, the SSX1 and SSX-2 genes were initially identified as fusion partners of the SYT gene in synovial sarcomas carrying *t*(X; 18)(p11; q11) translocations (Crew et al., 1995). This translocation leads to the expression of a chimeric protein whose function is still unclear, but likely act as

aberrant transcriptional regulator that might contribute to the malignant phenotype by inducing altered gene expression patterns (de Bruijn et al., 2002). SSX proteins are believed to function as transcriptional regulators, as supported by experimental data showing that SSX proteins were able to suppress the transcription of reporter genes (Brett et al., 1997; Lim et al., 1998; Thaete et al., 1999). Part of this activity was assigned to the N-terminal end of the SSX proteins, which exhibits extensive homology to the so-called Krüppel-associated box (KRAB), a domain that is present in a subgroup of zinc finger proteins and is involved in transcriptional repression (Margolin et al., 1994). However, considerably stronger repression was achieved by the most C-terminal portion, constituting the SSX Repression Domain (SSXRD) (Lim et al., 1998). More recently, yeast two-hybrid studies identified the human homologue of a Ras-like GTPase interactor (RAB3IP), and a novel nuclear protein, the SSX-2 interacting protein (SSX2IP), as binding partners for SSX-2 (de Bruijn et al., 2002). Despite these observed interactions may have important implications for the mechanisms underlying normal and malignant cellular growth, the way through which the SSX2IP and RAB3IP proteins modulate the functions of SSX2 has not been elucidated yet. Consistently, genes that might be potential targets for SSX2-mediated repression have not been defined to date.

In sharp contrast to our very limited knowledge on X-CTA function, most of the non-X CTA have known roles in spermatogenesis and fertilization. Among these are SCP-1, which is a part of the synaptonemal complex involved in chromosome pairing during meiosis (Tureci et al., 1998b), OY-TES-1 which acts in acrosin packaging in the acrosome of sperm heads (Ono et al., 2001), SPO11, a meiosis-specific endonuclease (Romanienko and Camerini-Otero, 1999) and the brother of the regulator of imprinted sites (BORIS), which is a recently described paralog of the epigenetic modulatory protein CCCTC-binding factor (CTCF), and is involved in the epigenetic reprogramming occurring during spermatogenesis (Loukinov et al., 2002). According to its physiologic role, recent data have implicated BORIS as playing a key role in promoting the expression of a number of CTA genes (Hong et al., 2005; Vatolin et al., 2005). Indeed, promoters of CTA genes are methylated and repressed in normal somatic cells expressing CTCF and not BORIS, but are specifically demethylated and activated in testis and in cancer cells that express BORIS. BORIS was demonstrated to bind directly CTA promoters and to displace CTCF at these loci (Hong et al., 2005; Vatolin et al., 2005). CTCF/BORIS binding sides were recently found in several MAGE-A genes that were co-expressed in head and neck cancer (Smith et al., 2009). Co-expression of BORIS with other CTA genes has been documented in several cancer cell lines and primary breast, prostate, colorectal and lung tumors tested (Hong et al., 2005; Vatolin et al., 2005). Ectopic BORIS overexpression was additionally shown to be sufficient to demethylate and activate 12 CTA genes in normal primary human fibroblasts (Vatolin et al., 2005). Kinetic studies reported that 5-AZA-CdR treatment activated BORIS expression within 6–8 h, whereas transcripts for MAGE-A1 were not detected until 48 h after of treating same cells with this demethylating agent (Vatolin et al., 2005). Furthermore, down-regulation of BORIS by RNA interference before 5-AZA-CdR treatment reduced the ability of 5-AZA-CdR to induce MAGE-A1 expression (Vatolin et al., 2005). A potential mechanism to explain the activating role of BORIS at CTA gene promoters is that it recruits the HMT SET1A as part of a transcriptional complex, thus promoting histone modifications associated with transcriptional activity (Nguyen et al., 2008). Altogether, these data provide intriguing evidence that aberrant expression of BORIS might have a role in regulating CTA expression in human cancer, but further studies will be necessary to clarify its function.

Although these molecular evidences globally address towards a potential implication of CTA expression in cancer development, the available literature data are initial and did not fully describe their role on cancer initiation and progression. Along this line, contrasting results reported both positive and negative impact of CTA expression on prognosis of cancer patients (Shigematsu et al., 2010; Atanackovic et al., 2009; Riener et al., 2009; Velazquez et al., 2007; Yoshida et al., 2006; Kim et al., 2006).

4. Therapeutic potential of CTA

4.1. *Immunogenicity of CTA*

CTA are widely expressed in tumors, but not in normal tissue except for testis that is not accessible to the immune system; in fact, the blood-testis barrier (Bart et al., 2002) and the lack of HLA class I expression on the surface of germ cells (Fiszer and Kurpisz, 1998) prevent the immune system from the interaction with CTA proteins to be recognized as non-self structures (Kalejs and Erenpreisa, 2005). Thus, from an immunological view-point, CTA may be regarded as essentially tumor-specific targets.

Distinct CTA encode for different antigenic peptides that are presented to the immune system in association with various HLA class I or HLA class II allospecificities (Traversari, 1999), eliciting both CTL and humoral immune responses (Jager et al., 1998). To date, spontaneous humoral and cell-mediated immune responses have been demonstrated against several CTA. In this context, NY-ESO-1 seems to be the most immunogenic CTA inducing spontaneous and coordinated humoral and cell-mediated immune responses in a high percentage of patients with NY-ESO-1 expressing tumors. Serum anti-NY-ESO-1 antibody has been detected in 36% of thyroid cancers (Maio et al., 2003b), 4%–12.5% of lung cancers (Stockert et al., 1998; Scanlan et al., 2001), 7%–13% of ovarian cancers (Scanlan et al., 2001; Stockert et al., 1998), 8%–16% of breast cancers (Stockert et al., 1998; Scanlan et al., 2001), 10% of melanomas (Stockert et al., 1998), 12.5% of bladder cancers (Kurashige et al., 2001) and 13% of esophageal cancers (Scanlan et al., 2001). Correlating with the antibody response, Jager et al. demonstrated that more than 90% of patients with circulating anti-NY-ESO-1 antibodies also developed an NY-ESO-1-specific CD8+ T-cell response that was absent in patients in whom anti-NY-ESO-1 antibodies were not detectable (Jager et al., 2000b).

4.2. *CTA vaccine therapy*

In light of the information above, it is clear that CTA represent ideal target molecules for specific immunotherapeutic

intervention in cancer patients. Accordingly, several clinical trials employing CTA, in particular MAGE-A3 and NY-ESO-1, as vaccinating agents in patients with lung, prostate, and ovarian cancers and melanoma are ongoing (Table 3) or have been completed (Slingluff et al., 2007; Brichard and Lejeune, 2007; Odunsi et al., 2007; Atanackovic et al., 2008; Bender et al., 2007; Valmori et al., 2007; Jager et al., 2006; van et al., 2005).

The characterization of immunogenic peptides from selected CTA, together with the identification of the respective HLA class I antigen restriction, represented the basis for the initial approaches of CTA-based immunotherapy of cancer patients, which relayed on the use of CTA peptides as vaccinating agents. In particular, MAGE-A3-derived peptides have been used as vaccines in HLA-A1-positive patients with tumor expressing the respective antigen. In a pivotal clinical trial with MAGE-A3 peptides in melanoma, 7 out of 25 patients showed significant tumor regressions, including 3 complete responses (Marchand et al., 1999). Despite these promising clinical results, MAGE-A3-specific CTL were not detected during the course of vaccinations, even in patients experiencing clinical responses (Marchand et al., 1999). In contrast to the poor immunological outcomes of MAGE-A3 peptide vaccination, intradermal immunization with NY-ESO-1 peptides was shown to induce primary NY-ESO-1-specific CD8+ T-cell responses. In a clinical trial with HLA-A2-restricted NY-ESO-1 peptides, Jager et al. reported that disease stabilization and regression of single metastases, induced in a patient with no previous spontaneous anti-NY-ESO-1 immunity, were associated with peptide-specific CD8+ T-cell responses. Furthermore, NY-ESO-1 antibody-positive patients did not develop significant changes in base-line NY-ESO-1-specific T-cell responses; however, stabilization of disease and regression of single metastases were observed in 3 out of 5 immunized patients (Jager et al., 2000a).

MAGE-A peptides have been also used in vaccines consisting of peptide-loaded monocyte-derived dendritic cells (DC). Nestle et al. treated 16 patients with metastatic melanoma with DC pulsed with HLA-A2-restricted tyrosinase, gp100 and melan-A peptides, and with HLA-A1-restricted MAGE-A1 and MAGE-A3 peptides. Five and 2 out of 16 patients experienced objective or complete tumor responses, respectively (Nestle et al., 1998). Along this line, Schuler et al. vaccinated HLA-A1-positive metastatic melanoma patients with 5 vaccinations of DC pulsed with an HLA-A1-restricted MAGE-A3 peptide. Significant expansions of MAGE-A3-specific CTL precursors were induced in 8 out of 11 patients. Regression of metastases was observed in 6 out of 11 patients, and T cell infiltrates were observed in a regressing metastasis (Thurner et al., 1999). These typical immunologic and clinical responses have been confirmed by additional trials in patients with melanoma, NSCLC, bladder and gastrointestinal carcinomas immunized with MAGE-A peptide-pulsed DCs (Banchereau et al., 2005; Escudier et al., 2005; Akiyama et al., 2005; Hersey et al., 2004; Palucka et al., 2003; Banchereau et al., 2001; Jonuleit et al., 2001; Mackensen et al., 2000; Morse et al., 2005; Sadanaga et al., 2001; Nishiyama et al., 2001).

Besides peptide vaccines, recombinant full-length MAGE-A3 and NY-ESO-1 proteins are currently being evaluated as anti-cancer vaccines in a series of clinical trials. Using recombinant proteins has many advantages, the more relevant being: i) the potential to induce both CD8+ and CD4+ immune responses; ii) the ability to generate concomitant immune responses against multiple epitopes; and iii) no requirement for a specific HLA type of patients to be treated. The first full-length CTA protein used to date for cancer patients vaccination has been a recombinant MAGE-A3 fusion protein, carrying a protein D domain of *Haemophilus influenzae* at its N terminus and a histidine (his) tag at its C terminus (Marchand et al., 2003). The first MAGE-A3 recombinant protein trial enrolled 32 MAGE-A3-positive patients with stage III or IV melanoma. This trial showed that MAGE-A3 recombinant protein administration was generally well tolerated and, among patients who received at least 4 vaccinations (26 patients), 1 partial response and 4 mixed responses were observed (Kruit et al., 2005). More recently, a phase II clinical trial (EORTC 16031-18032), in previously untreated patients affected by progressive unresectable stages III and IV M1a melanoma, was conducted. MAGE-A3-positive patients were randomized to receive MAGE-A3 protein in association with adjuvants AS02B or AS15. Objective responses and long-term disease stabilizations (>16 weeks) were observed in 5 out of 72 and 11 out of 72 patients, respectively. Anti-MAGE-A3 antibody titers were higher in the AS15 as compared to the AS02B arm, and CD4+ T-cell responses were observed in 72% and 36% of patients enrolled in the AS15 and AS02B arm, respectively (Kruit et al., 2008). In this trial, gene expression profiling, performed on tumor biopsies taken prior to any vaccination, revealed a correlation between clinical responses and the presence of a specific tumor molecular signature that could be prospectively used to select patients with a higher likelihood of clinical response to MAGE-A3 vaccines (Louahed et al., 2008). Based on these preliminary data, two large clinical trials have been lunched in MAGE-A3-positive cutaneous melanoma patients, both in the adjuvant (DERMA trial) and in the metastatic (PREDICT trial) setting. These studies will also prospectively validate the correlation between specific tumor molecular signatures and clinical responses. Besides cutaneous melanoma, adjuvant vaccination with MAGE-A3 recombinant protein was investigated in patients with MAGE-A3-expressing stage I or II NSCLC who had undergone surgical resection of the primary tumor. 9 patients received MAGE-A3 protein alone, whereas 8 patients received MAGE-A3 protein combined with adjuvant AS02B. Among patients who received MAGE-A3 protein alone, 3 developed an increase in the anti-MAGE-A3 antibody titer, and 1 developed a MAGE-A3-specific CD8+ T-cell response. By comparison, 7 and 4 out of the 8 patients who received MAGE-A3 protein combined with AS02B showed increased titers of circulating anti-MAGE-A3 antibodies and/or strong MAGE-A3-specific $CD4^+$ T-cell responses, respectively (Atanackovic et al., 2004). A subsequent phase II clinical trial of MAGE-A3 protein vaccine in 182 MAGE-A3-positive patients with stage IB or II NSCLC, revealed that the improvement of disease-free survival was 33% higher in MAGE-A3-receiving patients as compared to placebo (Brichard and Lejeune, 2007). Based on these preliminary activity data in melanoma, and on the promising phase II results in NSCLC, a phase III trial, which includes 2270 lung cancer patients, has been launched and is currently underway (Tyagi and Mirakhur, 2009). This study is a critical step for cancer

Table 3 – Ongoing clinical trials with CTA.

CTA	Sponsor	Tumor histotype	Phase	Combination	Identifier
MAGE-A3 (GSK2132231A[a])	Glaxo Smith Kline	Melanoma	I	Dacarbazine	NCT00849875[b]
MAGE-A3	University of Maryland	Head and neck	I	MAGE-A3 HPV[c]–16 vaccine	NCT00704041
MAGE-A10, NY-ESO-1, Melan-A	Ludwig Institute for Cancer Research	Melanoma	I	(1) Montanide + Melan-A analog peptide (2) Montanide + Melan-A analog peptide + NY-ESO-1 analog peptide + MAGE-A10 peptide (3) Montanide + CpG-7909/PF-3512676+Melan-A analog peptide + NY-ESO-1 analog peptide + MAGE-A10 peptide (4) Montanide + CpG-7909/PF-3512676 + Melan-A native and analog peptides + NY-ESO-1 long peptide + MAGE-A10 peptide (5) Montanide + CpG-7909/PF-3512676 + Melan-A native and analog peptides + NY-ESO-1 long peptide + MAGE-A10 peptide + low dose IL-2	NCT00112242
MAGE-A3 (GSK1572932A)	Glaxo Smith Kline	Lung	I	Cisplatin, vinorelbine, radiotherapy	NCT00455572
MART-1, tyr, gp100, MAGE-A3	Duke University	Melanoma	I	Proteasome siRNA and tumor antigen RNA-transfected dendritic cells	NCT00672542
NY-ESO-1	University of Pittsburgh	NY-ESO-1-expressing tumors	I	CpG 7909 and Montanide ISA 720 with or without cyclophosphamide in combination either with NY-ESO-1-derived peptides or the NY-ESO-1 protein	NCT00819806
NY-ESO-1, LAGE-1	Baylor College of Medicine	Prostate	I	NY-ESO-1 class I and class II peptide vaccine and LAGE-1 class I and class II peptide vaccine	NCT00711334
NY-ESO-1	Roswell Park Cancer Institute	Ovarian	I	ALVAC(2)-NY-ESO-1 (M)/TRICOM vaccine	NCT00803569
NY-ESO-1/gp100/MART-1	H. Lee Moffitt Cancer Center and Research Institute	Melanoma	I	Poly IC:LC, emulsified with Montanide ISA 51 with escalating doses of CP 870893	NCT01008527
NY-ESO-1	Roswell Park Cancer Institute	Ovarian	I	Incomplete Freund's adjuvant, sargramostim, decitabine, pegylated liposomal doxorubicin hydrochloride	NCT00887796
MART-1/gp100/NY-ESO-1	H. Lee Moffitt Cancer Center and Research Institute	Melanoma	I	MART-1:26–35 peptide vaccine, NY-ESO-1peptide vaccine, anti-PD-1 human monoclonal antibody MDX-1106, gp100:209–217 peptide vaccine, gp100:280–288 peptide vaccine, Montanide ISA 51 VG	NCT01176461
MAGE-A3, Melan-A, survivin	Dermatologische Klinik MIT Poliklinik-Universitaetsklinikum Erlangen	Melanoma	I, II	KLH, therapeutic autologous dendritic cells	NCT00074230
MAGE-A3, NA17.A2	Cliniques universitaires Saint-Luc-Université Catholique de Louvain	Melanoma	I, II	Vaccine MAGE-3.A1 peptide, or the NA17.A2 peptide + IL-2, IFN-α and GM-CSF, imiquimod.	NCT01191034
MAGE-A1/MAGE-A3, tyr/MART-1/gp100	Maria Sklodowska-Curie Memorial Cancer Center, Institute of Oncology	Melanoma	I, II	(1) HLA-A1-binding MAGE-A1/MAGE-A3 multipeptide-pulsed autologous dendritic cell vaccine (2) HLA-A2-binding tyr/MART-1/gp100 multipeptide-pulsed autologous dendritic cell vaccine (3) Autologous melanoma lysate-pulsed autologous dendritic cell vaccine (4) Autologous melanoma lysate/tracer antigen KLH-pulsed autologous dendritic cell vaccine (5) Dendritic cell-idiotype-KLH	NCT01082198
MART-1/gp100/tyr/NY-ESO-1	H. Lee Moffitt Cancer Center and Research Institute	Melanoma	I, II	Dendritic cell vaccine therapy, therapeutic autologous lymphocytes, fludarabine phosphate	NCT00313508
PRS pan-DR, MAGE-3 DP04, MAGE-1 A2, MAGE-3 A2, NY-ESO-1 A2 and MART-1 A2	Institut Gustave Roussy	Lung	II		NCT01159288

MAGE-A3 (GSK1203486A)	Glaxo Smith Kline	Melanoma	II		NCT00706238
gp100, MAGE-A3	M.D. Anderson Cancer Center	Melanoma	II	R848 gel	NCT00960752
MAGE-A3, NY-ESO-1	University of Arkansas	Multiple myeloma	II, III		NCT00090493
MAGE-A3 (GSK1572932A)	Glaxo Smith Kline	Lung	III	Placebo control	NCT00480025
MAGE-A3 (GSK2132231A)	Glaxo Smith Kline	Melanoma	III	Placebo	NCT00796445

a GSK, Glaxo Smith Kline product.
b Identifier of the trial as retrieved from: http://clinicaltrials.gov.
c HPV, Human Papilloma Virus; KLH, keyhole limpet hemocyanin; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN-α, interferon alfa; IL-2, interleukin 2; PD-1, programmed death 1; R848, imidazoquinoline resiquimod; siRNA, small interference RNA; tyr, tyrosinase.

vaccines, as it is the largest active cancer immunotherapy clinical trial to date.

As far as NY-ESO-1, the first clinical trial used a His-tagged recombinant full-length protein used alone or with a saponin-based adjuvant (ISCOMATRIX), to immunize NY-ESO-1-positive melanoma patients with minimal residual disease. All patients vaccinated with NY-ESO-1 ISCOMATRIX developed high titer anti-NY-ESO-1 antibody responses, whereas only 25% of those who received NY-ESO-1 protein without adjuvant did it. The vaccine also induced circulating CD4+ and CD8+ T cells specific for a broad range of NY-ESO-1 epitopes, including many previously unidentified ones (Davis et al., 2004). Noteworthy, only 2 out of 19 patients vaccinated with NY-ESO-1 ISCOMATRIX experienced tumor recurrence, in comparison to 14 out of 23 patients who received placebo or NY-ESO-1 alone (Davis et al., 2004). Recently, Nicholaou et al. examined the clinical and immunologic efficacy of NY-ESO-1 ISCOMATRIX vaccine on confirmed stage IV or unresectable stage III melanoma patients (Nicholaou et al., 2009). They found that, in contrast to minimal residual disease, advanced melanoma patients showed no clinical response to vaccination. Despite strong antibody responses were mounted, only 40% of patients had circulating NY-ESO-1-specific CD4+ T cells, and although 68% of patients showed CD8+ T cells specific to a broad range of NY-ESO-1 epitopes, the majority of these responses were preexisting. Compared with patients with minimal residual disease, patients with advanced melanoma were shown to have a significantly higher proportion of circulating regulatory T (Treg)-cells, which are able to strongly inhibit T cell activity, likely resulting in peripheral immunologic tolerance. These results pointed to a clear association among the stage of melanoma progression, the number of circulating Treg cells, and the clinical and immunologic efficacy of the NY-ESO-ISCOMATRIX anti-cancer vaccine (Nicholaou et al., 2009). So far, more than 30 clinical trials have been conducted with recombinant NY-ESO-1 worldwide, with different adjuvants and in different cancer types (Gnjatic et al., 2006). Most recently, an immunological analysis in metastatic melanoma patients previously treated with different therapeutic regimens (mostly chemotherapy), showed that the administration of the anti-CTLA-4 mAb ipilimumab enhanced polyfunctional NY-ESO-1-specific T-cell responses (Yuan et al., 2008). Of the 15 patients receiving ipilimumab, 8 showed evidence of clinical benefit in terms of complete or partial responses, or stable disease. Of these, 5 were found to be NY-ESO-1 antibody positive, whereas none of 7 clinical non-responders had anti-NY-ESO-1 circulating antibodies. All the 5 patients with NY-ESO-1 antibodies had clearly detectable CD4+ and CD8+ T cells against NY-ESO-1 following treatment with ipilimumab (Yuan et al., 2008). These evidences clearly suggest that ipilimumab therapy and anti-NY-ESO-1 vaccination might have a synergistic effect, thus warranting further studies.

4.3. *Combinatorial use of CTA and epigenetic drugs*

Despite the promises of CTA-based vaccination, the inter- and intratumoral heterogeneous expression of CTA might: i) limit the biological eligibility of cancer patients to treatment; ii) impair immunogenicity and immune recognition of neoplastic

cells thus reducing the efficacy of vaccination; or iii) lead to the emergence of CTA-negative neoplastic clones, escaping treatment-induced CTA-specific immune surveillance. In this context, the well-characterized role of promoter methylation in regulating CTA expression has suggested the possibility to therapeutically modulate CTA expression in neoplastic cells, through the administration of DHA (De Smet et al., 1999; Sigalotti et al., 2002b, 2004; Yuan et al., 2008). Along this line, *in vitro* evidences proved the effectiveness of the DHA 5-AZA-CdR to induce and/or to up-regulate the concomitant expression of multiple members of different CTA families (e.g., MAGE-A, NY-ESO, GAGE) in neoplastic cells of different histotypes. Importantly, these phenotypic modifications were functional, inducing or potentiating the recognition of neoplastic cells by CTA-specific CTL (Coral et al., 2002; Sigalotti et al., 2004, 2002a; Roman-Gomez et al., 2007a, b; Adair and Hogan, 2009). These observations were further expanded by Sigalotti et al., who demonstrated, through an *ex vivo* model, that 5-AZA-CdR is also able to revert the intratumoral heterogeneous expression of CTA, leading to a homogeneous targeting of neoplastic cells by MAGE-A-specific CTL (Sigalotti et al., 2004). An important aspect shared by the above reported phenotypic modifications induced by DHA is that they are persistently retained by the neoplastic cell population (Sigalotti et al., 2004; Weber et al., 1994; Calabro et al., 2005; Coral et al., 1999). This key behavior is clearly inline with the physiologic inheritance of DNA methylation patterns, and has been recently demonstrated to rely on the single-cell level heritability of CTA promoter demethylation induced by 5-AZA-CdR treatment (Fratta et al., 2010).

Besides their direct action on CTA expression, DHA are characterized by a more general immunomodulatory activity, which includes the persistent up-regulation of different molecules involved in antigen presentation machinery, such as HLA class I antigens and accessory molecules, expressed on neoplastic cells (Coral et al., 1999). The functional importance of this property has been recently defined by Fonsatti et al., who demonstrated that the sole modulation of HLA class I antigens and of CD54 on melanoma cells was sufficient to significantly increase their recognition by antigen-specific CTL (Fonsatti et al., 2007). Accordingly it could be anticipated that this broader activity of DHA would synergize with CTA modulation, giving a further contribution in the improvement of CTA-specific anti-tumor immune recognition.

The demonstrated *in vitro* immunomodulatory activities of 5-AZA-CdR were further evaluated in pre-clinical animal models to assess for their potential clinical transferability. Along this line, recent data reported that systemic administration of 5-AZA-CdR in BALB/c mice grafted with the murine mammary carcinoma cells 4T1 induced the expression of the murine CTA P1A in tumors, and determined a significant reduction in the number of 4T1-derived lung metastases upon adoptive transfer of P1A specific CTL (Guo et al., 2006). Furthermore, *in vivo* administration of 5-AZA-CdR in BALB/c *nu/nu* mice grafted with primary cultures of human cutaneous melanoma cells, persistently induced and/or up-regulated the expression of several CTA and up-regulated HLA class I antigens in the neoplastic tissues (Coral et al., 2006, 2007). Noteworthy, the phenotypic changes induced by 5-AZA-CdR on neoplastic cells have been shown to potentiate their *in vivo* immunogenicity, as demonstrated by the generation of high titer circulating antibodies against the *de novo* induced NY-ESO-1 protein in BALB/c mice immunized with 5-AZA-CdR-treated human melanoma cells (Coral et al., 2006).

Despite the significant amount of pre-clinical data demonstrating an important immunomodulatory activity of DHA both *in vitro* and *in vivo*, to date this aspect has been only marginally addressed in human experimentations. Even so, the few initial studies that have evaluated the activity of 5-AZA-CdR on CTA expression in patients provided encouraging results. The first study demonstrating an immunomodulatory activity of 5-AZA-CdR in humans was conducted on patients with hematologic malignancies. In this setting, 5-AZA-CdR was demonstrated to induce a persistent *de novo* expression of MAGE-1, SSX and NY-ESO-1 antigens in peripheral blood and bone marrow mononuclear cells of patients affected by AML or MDS, treated with a single course of systemic 5-AZA-CdR (Sigalotti et al., 2003). Whether the immune recognition of the *de novo* expressed CTA might contribute, at least in part, to the long-term disease control observed in selected AML/MDS patients still remains to be addressed, but it certainly represents an appealing hypothesis. This immunomodulatory activity of DHA seems not to be restricted to hematologic malignancies. Indeed, a recent phase I trial, investigating systemic 5-AZA-CdR in patients with thoracic malignancies, demonstrated that a molecular response, consisting in the *de novo* expression of NY-ESO-1, MAGE-A3, or p16, was achieved in the tumor tissue of 8 out of 22 patients available for analysis. Interestingly, post-treatment antibodies to NY-ESO-1 were detected in 3 patients exhibiting induction of NY-ESO-1 in their tumor tissues, demonstrating that the *de novo* expressed CTA can be readily recognized by the host's immune system and could represent promising therapeutic targets (Schrump et al., 2006).

5. Conclusions

In the last decades, CTA have emerged as important cancer therapeutic targets based on their tumor-restricted expression pattern, immunogenicity and putative role in oncogenesis. Vaccines targeting distinct CTA have been developed so far, and these approaches have shown to promote immune and clinical responses. The final clinical success of CTA-based vaccines, however, is clearly dependent on the deployment of optimal strategies to comprehensively direct the immune system against the tumor cells. In this respect, the recent advances in the knowledge of the fine-tuning mechanisms of the immune response are providing new therapeutic tools that are able to globally boost the performance of the immune system. Immunomodulatory antibodies, in particular, are emerging as a class of agents that are highly effective in triggering activation of T cells, leading to important clinical results (Melero et al., 2007). Among these, the anti-CTLA-4 mAb ipilimumab was recently described to enhanced polyfunctional NY-ESO-1-specific T-cell responses in treated melanoma patients (Yuan et al., 2008), suggesting that combined therapies including immunostimulatory antibodies and CTA-based vaccines could be undertaken to achieve better clinical results. On the other

hand, the ability of epigenetic drugs to restore the defective expression of CTA, to homogenize their intratumoral distribution, as well as to up-regulate HLA antigens and accessory/co-stimulatory molecules, prompts for their use to widen biologic eligibility of patients to CTA-based vaccination, as well as to render cancer cells more immunogenic and less prone to evading immune recognition. Comprehensively a wide range of appealing combination therapies awaits to be explored to improve the effectiveness of CTA-based immunotherapeutic approaches.

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